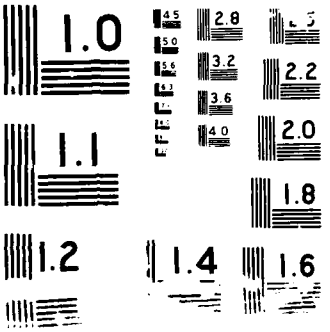
AD-A194 391 MEMBRANE ALTERATIONS FOLLOWING TOXIC CHEMICAL INSULT (U) STATE UNIV OF NEW YORK AT BINGHAMTON DEPT OF BIOLOGICAL SCIENCES A LISS 10 MAR 88 AFOSR-TR-88-0460 1/1

UNCLASSIFIED AFOSR-84-0153 F/G 6/11 NL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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS: NONE
2a. SECURITY CLASSIFICATION AUTHORITY:	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE:	
AD-A194 391	5. MONITORING ORGANIZATION REPORT NUMBER(S): AFOSR-TR- 88-0460
6a. NAME OF PERFORMING ORGANIZATION: University Center at Binghamton State University of New York	6b. OFFICE SYMBOL (If applicable):
6c. ADDRESS (City, State, and ZIP Code): Biology Department Binghamton, NY 13901	7a. NAME OF MONITORING ORGANIZATION: Air Force Office of Scientific Research/NL
	7b. ADDRESS (City, State, and ZIP Code): Building 410 Bolling AFB, DC 20332-6448
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: AFOSR	8b. OFFICE SYMBOL (If applicable): NL
	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: AFOSR-84-0153
8c. ADDRESS (City, State, and ZIP Code): Building 410 Bolling AFB, DC 20332	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 61102F; PROJECT NO. 2312; TASK NO. A5; WORK UNIT ACCESSION

11. TITLE (Include Security Classification): Membrane Alterations Following Toxic Chemical Insult

12. PERSONAL AUTHOR(S): Alan Liss

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
FINAL	FROM 7/15/84 TO 1/31/88	1988, MARCH 10	9 + i

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	in vitro toxicology, perfluorinated fatty acids, mycoplasmas, membranes

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

A procaryotic cell system was developed that can be used to determine the toxic action of chemicals acting at the level of the eucaryotic or procaryotic cytoplasmic membrane. Cell wall-less micobes known as mycoplasmas were used. In this current study, two perfluorinated fatty acids (C8 and C10) were found to inhibit the growth of the test mycoplasmas. Two apparent activities, cytotoxicity and cytolysis, were observed. At high concentrations (>10 mM) a detergent-like action was noted. At low concentrations (<10 mM) cell death was observed without detectable cell lysis. Altering the cell membrane (the presumed target of the toxic compounds) resulted in altered levels of toxicity. Similar results were obtained when human or murine B-cells were used as the target organism. The toxic action of the perfluorinated fatty acids apparently involves some interaction with the membrane of the cells being treated.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Major T. Jan Cerveny	22b. TELEPHONE (Include Area Code): (202) 767-5021
	22c. OFFICE SYMBOL: NL

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

MEMBRANE ALTERATIONS FOLLOWING TOXIC CHEMICAL INSULT - AFOSR - 84- 0153

YEAR 3 RESEARCH PROGRESS REPORT AND FINAL TECHNICAL REPORT

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ABSTRACT

A procaryotic cell system was developed that can be used to determine the toxic action of chemicals acting at the level of the eucaryotic or procaryotic cytoplasmic membrane. Cell wall-less micobes known as mycoplasmas were used. In this current study, two perfluorinated fatty acids (C8 and C10) were found to inhibit the growth of the test mycoplasmas. Two apparent activities, cytotoxicity and cytolysis, were observed. At high concentrations (>10 mM) a detergent-like action was noted. At low concentrations (<10 mM) cell death was observed without detectable cell lysis. Altering the cell membrane (the presumed target of the toxic compounds) resulted in altered levels of toxicity. Similar results were obtained when human or murine B-cells were used as the target organism. The toxic action of the perfluorinated fatty acids apparently involves some interaction with the membrane of the cells being treated.

INTRODUCTION

The primary goal of this grant proposal was to investigate the toxic modality of perfluorinated fatty acids. The target cells for this study were the cell wall-less procaryotes commonly called mycoplasmas. Using this interesting procaryotic cell system, a secondary goal was to establish an *in vitro* system for identifying membrane active toxicants acting on both procaryotic and eucaryotic cells. After a period of three years, the primary goal has been accomplished and the secondary goal is well within reach.

A large number of environmentally important compounds exist. In many cases, we learn of the toxic nature of these compounds after they appear in the environment. Often this "after the fact" recognition of toxicity is due to the unavailability of rapid and cost effective toxicity model systems. Extensive model systems exist for determining the toxic action of certain chemicals of a cell's DNA. However, the toxicity of many compounds is predicated on the initial interaction of the compound with the cell's outermost structure- the cytoplasmic membrane.

Halogenated compounds play a large part in todays industrialized world. These compounds are used as refrigerants, fire retardants, paint and varnish components, and solvents. Many of these halogenated compounds are also commonly used pesticides and herbicides. Besides the known toxicity of these latter noted compounds, most halogenated compounds can generally be considered overt or at least highly suspect toxins (Ghosal, *et al*, 1985).

My current studies involve using mycoplasmas, cell wall-less procaryotes (Tully and Razin, 1977, Razin, 1985), as the sentinel organisms to determine the toxic action of perfluorinated hydrocarbons. Unlike typical eubacteria, the mycoplasmas are bounded solely by a unit biological membrane. In addition, they lack any internal membrane systems. Many studies involving structure/function relationships in biomembranes have been carried out using mycoplasmas as "model" membrane systems (*cf* Archer,1981). Similar to eucaryotic cells, mycoplasmas incorporate sterols into their membranes. In fact, several species of mycoplasmas are the only procaryotes known to have an absolute requirement for cholesterol. I am using three species of two different mycoplasma genera in this study. *Acholeplasma laidlawii* is a sterol non-requiring mycoplasma. It is one of the least fastidious of these microbes. Extensive studies have been carried out with the membranes of *A. laidlawii*. The final composition of their cellular membranes is decided by genetic as well as environmental factors. *A. laidlawii* can be infected with several acholeplasmaviruses that are released without lysis of the infected cell (rev. in Razin, 1985). This infection process is altered by changing the composition of the host membrane,

presenting an interesting model for a biological process involving the cell membrane. Two members of the sterol requiring mycoplasmas (genus Mycoplasma) are also being used in this study. These species, M. capricolum and M. gallinarum, are more fastidious than A. laidlawii and they incorporate up to 10 times as much cholesterol in their membranes (Bittman et al, 1983). This compositional difference presents a biological membrane with different biophysical constraints as compared to those for A. laidlawii. In addition, although both M. capricolum and M. gallinarum are lysed by digitonin (presumably acting upon the cholesterol in the cell membrane, Razin and Argeman, 1963) only M. capricolum is sensitive to sodium polyethanol sulfate (also believed to interact with cholesterol, Mardh, 1975). These three mycoplasmas represent three similar yet different microbes with membranes of definable character which can be tested against toxins with possible membrane targets.

I have been studying the perfluorinated straight chain hydrocarbons, nonadecafluoro-n-decanoic acid (NDFDA) and pentadecafluoro-n-octanoic acid (PFDA). It is clear from my results that these compounds act upon the membrane of cells and that this target is the same in both procaryotic and eucaryotic cells.

Personnel Involved

Alan Liss, Ph. D., Assistant Professor, Pricinple Investigator
Joanne Pfeil, M.S., Research Associate
Jomary Rojas, B.S., Graduate Student
Anne Vucic, B.S., Graduate Student
Brenda E. Ritter, Undergraduate Student

Publications and Presentations

PUBLICATIONS

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4) Liss, A., Pfeil, J. C. and Levitt, D.: Perfluorinated fatty acid is toxic for both eucaryotic and procaryotic cell sysytems. Sixteenth Conference on Toxicology, Dayton, OH. 1986.

SUMMARY OF STUDIES

BIOLOGICAL PARAMETERS

Organisms Used

The sentinel organisms used in this model, Acholeplasma laidlawii, Mycoplasma capricolum, and M. gallinarum, were cultivated in a Bacto tryptose-based growth medium supplememted with either 1 % PPLO serum fraction (Difco Labs, Detroit, MI) (for A. laidlawii) or 10 % donor horse serum (K C Biologicals, Lenexa, KA) (all strains). Although other differences exist between these two serum supplements, the major difference appears to be that of cholesterol content. Enzymatic cholesterol assays (Sigma Chemicals, St. Louis. MO) showed that Mycoplasma sp. grown under these conditions have a relative amount of cholesterol 5 times (using arbitrary units) that of Acholeplasma laidlawii grown in the same horse serum containing medium. In addition, A. laidlawii grown in horse serum had approximately 10 times the cholesterol observed when grown in serum fraction supplemented medium.

Growth Inhibition on Agar

Using 0.6 cm (diameter) sterile filter paper discs loaded with 25 ul of 100 mM of NDFDA, growth inhibition of the two mycoplasmas was less than 1 cm as compared to cleared zones of 2.0 cm or more when A. laidlawii was the test organism. At similar concentrations, no inhibitory zones were noted when capric acid was tested on A. laidlawii or M. capricolum. A small inhibitory zone (< 1 mm) was observed when the test organism was M. gallinarum. These tests helped to determine the concentrations of toxicants to be used in broth culture experiments as discussed below.

Recently, this simple protocol has been used to test the interactions of combinations of several presumptive toxicants on our target cells. The compounds hematoporphyrin (HP) and sodium selinite (SS) were used in combination with NDFDA. It was observed after incubation at 37C for 20 hrs in the presence of light, that all three compounds inhibited A. laidlawii growth 1.5 cm or more (concentrations of 1.0 mg/ml for HP, 1.0 M for SS, and 100 mM for NDFDA) when tested separately. However, when mixed (in any combination) binary solutions did not inhibit the test organism to the same extent.

Growth Inhibition in Broth

A test was constructed that would establish the concentration of test compound that would kill (i.e. eliminate colony forming ability) a standard concentration of the test organism after 30 min at 37C. Starting with a 10 mM solution of test compound, serial two-fold dilutions were made with a buffer containing 10^6 colony forming units (CFU) per ml of test organism. Samples were removed at time zero and then 30 minutes later and plated onto the appropriate agar medium. After incubation at 37C for 3 to 5 days, CFU were counted. The minimum concentration that would reduce the detectable CFU to zero at a 100-fold dilution of the initial mixture was defined as the minimum toxic concentration of the test compound. For these studies, capric acid and NDFDA, only, were used.

For Acholeplasma laidlawii propagated under low cholesterol growth conditions (supplemented with serum fraction), the minimum inhibiting concentration of capric acid and NDFDA were determined to be 2.5 mM and 0.5 mM , respectively. When A. laidlawii was supplemented with horse serum (which increases the cholesterol content in the membranes) the minimum inhibitory concentrations were observed to be 5.0 mM for capric acid and 2.5 mM for NDFDA (Liss et al, 1987).

Similar test using M. gallinarum and M. capricolum were performed. Both organisms were grown only in horse serum supplemented medium (defined as high cholesterol conditions). The established minimum toxic concentration of both capric acid and NDFDA was the same (2.5 mM) when M. capricolum was the test organism. When M. gallinarum was the test organism, it appeared that capric acid was more toxic than was NDFDA (5.0 mM as compared to 2.5 mM) (Liss et al, 1987).

Acholeplasmavirus Studies

A. laidlawii can be productively infected with four different types of viruses (Razin, 1985). Progeny of each virus is released without lysis of the infected cell. The kinetics of virus release are affected by the membrane composition of the host cell (Steinick et al, 1980). The test compounds used in this study were tested for the ability to alter viral growth patterns. At sub-toxic concentrations of NDFDA (0.4 mM) virus release was more rapid than that seen in control samples. This may have breen an artefact as the same concentration of NDFDA released "clumps" of viruses in cell free solutions. At higher concentrations (0.5 to 0.7 mM), virus release is inhibited after treatmentment for 30 min. This inhibition is "reversible" if cells are removed from the toxicant within 30 min of treatment and moved to NDFDA free medium for 60 min. The mechanism involved in this response is still undefined. Capric acid had no effect at similar concentrations.

Tests Using Human and Murine B-cells

One key element of this model is that it supplies information of predictive value relevant to eucaryotic as well as procaryotic cells. To test this we (in collaboration with Daniel Levitt, Guthrie Research Institute, Sayre, Pa) treated human and murine B-cells following protocols established with the mycoplasma model. Again, we observed that NDFDA had a cytotoxic and a cytolytic activity, depending on concentration (Levitt and Liss, 1986). Using a lipophilic dye,merocyanine 540, it was found that NDFDA treatment altered the interaction of dye and its membrane target - phospholipids (Levitt and Liss, 1987). We concluded that these data confirmed the membrane level (and probable hydrophobic nature) of the NDFDA cell target.

BIOCHEMICAL PARAMETERS

Polypeptide Profile Analyses

Preliminary studies in a broth culture growth system revealed that two actions of the perfluorinated fatty acids can be discerned. At concentrations of NDFDA greater than 10 mM, the A. laidlawii cells were actually solublized (e.g. the turbidity of the cell-toxin mixture decreased). Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that polypeptides were lost from the toxin treated whole cells when compared to the untreated cells. In general the solubilization was not specific for any special fraction of the total cellular polypeptides. The changes in SDS-PAGE profiles were similar to those seen when a detergent such as sodium dodecyl sulfate was used to treat the cells. In addition, alterations in polypeptide profiles due to treatment with the perfluorinated fatty acids were not similar to those seen when proteinase K was used to treat the cells (Liss et al, 1987).

Treating the cells with high concentrations of the non-perfluorinated fatty acid control chemicals produced similar "detergent-like" activity. Note that this result differs from that described in regard to the cytotoxic nature of these compounds, as given above. Using the same concentrations of NDFDA and PFOA, M. capricolum and M. gallinarum polypeptide profiles were also changed as if treated with a classical detergent (Liss et al, 1987).

Hematoporphyrin (HP), in the presence of light, has been shown to cross-link the polypeptides of the target mycoplasma cells (Rojas, unpublished data). When binary mixtures of NDFDA and hematoporphyrin were used to treat cells, this cross linking was inhibited at concentrations previously shown to be active. These studies are being continued to attempt to understand the mechanisms behind these observed events.

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